ANTI-PLASMODIUM COMPOSITIONS AND METHODS OF USE

Field of the Invention

The present invention relates to the fields of microbiology and immunology and more specifically relates to compositions and methods for the detection, diagnosis, prevention and treatment of malaria. In particular, the invention pertains to a family of paralogues of EBA-175, antibodies specific to each paralogue, peptides of the paralogues and peptides of the antibodies that inhibit the binding of *Plasmodium falciparum* erythrocyte binding protein antigens to erythrocytes.

Background of the Invention

Although endemic malaria has disappeared from the United States, malaria continues to be one of the most important infectious diseases in the world as it kills millions of people each year in countries throughout Africa, Asia and Latin America. The characteristic presentation of malaria is chills followed by a fever ranging from 104-107°F, followed by profuse sweating. Other manifestations of malaria include

anemia, decreased blood flow to vital organs, thrombocytopenia, and glomerulonephritis. Additionally, when the central nervous system is involved, symptoms include delirium, convulsions, paralysis, coma, and even rapid death.

Malarial diseases in humans are caused by four species of the *Plasmodium* parasite: *Plasmodium falciparum* (*Pf*), *Plasmodium vivax* (*Pm*), *Plasmodium ovali* (*Po*), and *Plasmodium malariae* (*Pm*). Each of these species is transmitted to the human via a female *Anopheles* mosquito that transmits *Plasmodium* parasites, or sporozoites. Once the sporozoites enter the bloodstream of the human, they localize in liver cells, or hepatocytes. One to two weeks later, the infected hepatocytes rupture and release mature parasites, or merozoites. The merozoites then begin the erythrocytic phase of malaria by attaching to and invading red blood cells, or erythrocytes.

The invasion of the erythrocytes by the malarial parasites is the direct cause of malarial pathogenesis and pathology. The fever, anemia, circulatory changes, and immunopathologic phenomena characteristic of malaria are largely the result of red cell rupture and the host's immune response to parasitized erythrocytes. For these reasons, the erythrocytic stage of the *Plasmodium* life cycle is of vital importance to vaccine development and treatment of malaria.

There are a number of strategies for developing new or novel therapeutics for the erythrocytic stage of malaria. One strategy is to identify parasitic molecules that are critical to the survival of the parasite. Extracellular merozoites released from infected hepatocytes or from infected erythrocytes must invade other erythrocytes within minutes if they are to survive. Invasion by the malaria parasite is dependent upon the binding

of parasite proteins to receptors on the erythrocyte surface (Hadley *et al.*, 1986).

Interestingly, different parasite species use different erythrocytic receptors for invasion of erythrocytes. *P. falciparum* invades erythrocytes through a 175 kDa erythrocyte binding protein called EBA-175. The gene encoding EBA-175 of Pf has been cloned and sequenced (Sim *et al.*, 1990; Fang *et al.*, 1991). EBA-175 functions as an erythrocyte invasion ligand that binds to its receptor, glycophorin A, on erythrocytes during invasion (Camus and Hadley, 1985; Sim *et al.*, 1990; Orlandi *et al.*, 1992; Sim *et al.*, 1994b). In contrast, the human *P. vivax* and the simian *P. knowlesi* invade erythrocytes by binding Duffy blood group antigens present on some erythrocytes (Miller *et al.*, 1975). The genes encoding the Duffy antigen binding proteins of *P. vivax* and *P. knowlesi* have been cloned and sequenced (Fang *et al.*, 1991 and Adams *et al.*, 1990, respectively).

Sequencing of the genes encoding the proteins used by *P. vivax* and *P. knowlesi* for erythrocyte invasion demonstrated that these proteins are members of the same gene family as the genes that encode the EBA-175, the protein used by *P. falciparum* for erythrocyte invasion (Adams *et al.*, 1992). Homology between the Duffy binding proteins and EBA-175 is restricted to 5' and 3' cysteine rich domains. Within these cysteine rich domains, the cysteines and some aromatic residues are conserved, but the intervening amino acid sequences differ. Sim *et al.* (1994b) demonstrated that the 5' cysteine rich domain of EBA-175 of *P. falciparum* contains the receptor binding domain, while Chitnis and Miller (1994) demonstrated that the 5' cysteine rich region of *P. vivax* and *P. knowlesi* contain the Duffy binding domain. See Figure 1.

What is needed for erythrocytic malaria vaccine development is the identification and targeting of parasite molecules involved in the process of erythrocyte invasion. Blockade of this ligand-receptor-mediated event can inhibit parasite development *in vitro*.

Invasion of erythrocytes by malaria parasites results from merozoite ligands interacting with erythrocyte receptors. Within *Plasmodium* a family of merozoite ligands (orthologues) that are erythrocyte-binding proteins (EBPs) has been identified. In the most severe human malaria, *Plasmodium falciparum*, the EBP is identified as the erythrocyte binding protein-175 (or EBA-175), and in*P. vivax*, the second most prevalent human malaria and the simian malaria *P. knowlesi*, the EBPs are termed the Duffy antigen binding proteins (PvDABP or PkDABP, respectively). Analysis of the deduced amino sequence of these orthologous proteins (EBA-175, PvDABP and PkDABP) lead to the classification of seven distinct domains encoding regions of greater or lesser similarity (Adams *et al.* 1992). Within the molecular family, two regions show significant levels of conservation, these are identified as region II (RII), which encodes for the ligand-binding domain and region VI (RVI), which has as yet an unknown function. EBA-175 RII binds sialic acid residues in conjunction with the peptide backbone of glycophorin A. PvDABP and PkDABP RII bind the Duffy blood group antigens, which are chemokine receptors(Horuk *et al.* 1993). PvDABP and PkDABP have a single cysteine rich domain (termed F1) while EBA-175 RII contains essentially a duplicate of F1, termed F1 and F2. Both RII and RVI domains contain cysteine rich motifs. One function of RII and RVI is apparently to provide for a conserved tertiary structure, critical for the ligand-receptor interaction that leads to erythrocyte

invasion. Erythrocytic invasion by *P. vivax* merozoites appears dependent on the Duffy blood group antigens while in vitro studies have shown that *P. falciparum* may use alternative invasive pathways (Narum *et al.* 2000, Dolan *et al.* 1994).

Summary of the Invention

The present invention provides compositions and methods for detecting, diagnosing, preventing and treating *Plasmodium falciparum* and *Plasmodium falciparum* related infections. In particular, the compositions include a family of merozoite ligands that are erythrocyte binding proteins (EBPs). The EBPs of the invention are paralogues of EBA-175, an EBP in *P. falciparum*. The invention further comprises antibodies specific to each paralogue, peptides of the paralogues, and peptides of the antibodies that specifically inhibit binding of *P. falciparum* erythrocyte binding proteins to erythrocytes.

The cysteine residues of the EBPs are conserved within the erythrocyte binding domain of EBA-175 RII. Some of the family members of the present invention include EBP2 (SEQ ID NO:1), EBP3 (SEQ ID NO:2), EBP4 (SEQ ID NO:3), and EBP5 (SEQ ID NO:4).

More specifically, the paralogues and respective peptides of the invention are useful as potential vaccine candidates, targets for producing antibodies, targets for small blocking peptides, and diagnostics.

The compositions also include *P. falciparum* antibodies to the family of EBA-175 paralogues and antibody fragments thereto, *P. falciparum* blocking peptides derived from the paralogues and/or antibodies thereto, *P. falciparum* antisera, *P. falciparum* receptor agonists and *P. falciparum* receptor antagonists linked to cytotoxic agents. Such compositions are also useful for research applications, as

vaccine candidates, as blocking peptides, diagnostics and prognostics. The compositions, when combined with pharmaceutically acceptable excipients, or sustained-release compounds or compositions, such as biodegradable polymers, are useful as therapeutic agents such as vaccine or treatment compositions.

Diagnostic and analytical methods and kits may be developed for detection and measurement of *P. falciparum* in a variety of biological samples including biological fluids and biological tissues, and for localization of *P. falciparum* in tissues and cells. The method and kit can be in any configuration well known to those of ordinary skill in the art.

The methods of the present invention include methods of treating, diagnosing and preventing *P. falciparum* diseases such as malaria. These methods employ the family of paralogues of the EBA-175 protein, the antibodies specific for individual paralogues and the blocking peptides described herein. Methods of prevention may include passive immunization prior to infection by *Plasmodium falciparum* parasites to inhibit parasitic infection of erythrocytes. Methods of treatment may also include administration after infection to inhibit the spread of the parasite and ameliorate the symptoms of *P. falciparum* infection. Methods of diagnosis of *P. falciparum* infection include methods directed toward combining a biological sample with the paralogues and/or antibodies described herein, wherein the binding of the paralogues and/or antibodies indicates malaria. Methods of detection of *P. falciparum* and *P. falciparum* erythrocyte binding proteins include methods directed toward the detection of *P. falciparum* and *P. falciparum* erythrocyte binding proteins or antibodies thereto in biological samples such as biological fluids, tissues and in culture media.

Also provided are methods of detecting additional family members of the paralogues of *P. falciparum* and *P. falciparum* erythrocyte binding proteins. Criteria include the identification of regions within malaria proteins that likely share a molecular structure that function as a receptor binding domain as identified by homology between EBP region II (F1 and/or F2) and/or region VI. The ligand binding region of interest is primarily the disulfide forming amino acid cysteine rich region which also includes other aromatic amino acids such as proline and tryptophan.

Accordingly, it is an object of the present invention to provide compositions comprising one or more paralogues of *P. falciparum* erythrocyte binding protein, antibodies specific for each paralogue or blocking peptides derived from the paralogues or antibodies.

It is another object of the present invention to provide a method for the treatment of *P. falciparum* related diseases such as malaria.

It is a further object of the present invention to provide a method for the treatment of malaria, wherein compositions comprising one or more paralogues of *P. falciparum* erythrocyte binding protein, antibodies specific for each paralogue and/or blocking peptides derived from the paralogues or antibodies are administered to an individual in need of such treatment.

It is another object of the present invention to provide a method for the diagnosis of *P. falciparum* related diseases such as malaria.

It is yet another object of the present invention to provide a method for the diagnosis of malaria, wherein compositions comprising one or more paralogues of *P.*

falciparum erythrocyte binding protein, and/or antibodies specific for each paralogue are used.

A further object of the present invention is to provide a method for the prevention of *P. falciparum* related diseases such as malaria.

It is another object of the present invention to provide a method for the prevention of malaria, wherein compositions comprising one or more paralogues or regions thereof of *P. falciparum* erythrocyte binding protein, antibodies specific for each paralogue and/or blocking peptides derived from the paralogues or antibodies are administered to an individual in need of such prevention.

Another object of the present invention to provide a method of detection of *P. falciparum* in culture media and in biological samples such as biological tissues and fluids.

It is a further object of the present invention to provide a method of detection of *P. falciparum*, wherein compositions comprising one or more paralogues of *P. falciparum* erythrocyte binding protein and/or antibodies specific for each paralogue are used.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Brief Description of the Figures

Figure 1: *Gene Structure of Plasmodium Erythrocyte Binding Proteins*

Region I of EBA-175 encompasses amino acid residues 20-157, region II amino acids 145-760, region III-V

amino acids 743-1322 and region VI amino acids 1304-1394. Region II is further subdivided into regions F1 and F2.

Figure 2: *Nucleotide and deduced amino acid sequence alignment of five EBA-175 Blocking Peptides*

Nucleotide and deduced amino acid sequence alignment of partial ORF of *eba-175 (ebp1), ebp2, ebp3, ebp4 and ebp5*. Identical amino acid residues are shown in boxes.

Figure 3: *EBP2 specific polyclonal antibodies blocked EBP2 binding to human erythrocytes*

The blocking of binding of processed fragments of EBP2 with erythrocytes as a percent compared to the control which was determined at a 1/10 dilution. Equal volumes of erythrocyte lysate were immunoprecipitated with EBP2 specific polyclonal antibodies.

Detailed Description

Compositions and methods for preventing and treating *P. falciparum* infection, diagnosing diseases related to *P. falciparum* infection, and preventing diseases related to *P. falciparum* infection are provided. The compositions include at least one of a family of paralogues, antibodies specific for each paralogue and peptides derived from the paralogues and antibodies that specifically inhibit binding of *P. falciparum* erythrocyte binding proteins and fragments thereof. The paralogues, antibodies and fragments thereof are useful in malaria vaccines and for receptor blocking therapies.

More specifically, a family of paralogues to the EBA-175 erythrocyte binding protein (EBP) has been identified in *P. falciparum*. The cysteine residues of the EBPs are conserved within the erythrocyte binding domain of EBA-175

RII. The RII region was previously disclosed in U.S. Provisional Patent Application Nos. 60/122,842, 60/153,575, PCT/US00/05820, and U.S. Pat. Nos. 5,993,827 and 5,849,306 to Sim *et al.*, each of which is herein incorporated by reference. Examples of the family members of the present invention are shown in Fig. 2 and include EBP2 (SEQ ID NO:1), EBP3 (SEQ ID NO:2), EBP4 (SEQ ID NO:3), and EBP5 (SEQ ID NO:4). Fig. 2 is an alignment report of EBP1_5 070500.MEG, using Clustal method with PAM250 residue weight table.

Characteristics of the family members include conservation of the cysteine rich domains identified as RII (F1 and/or F2), and/or RVI described herein. Members of this family are chosen by the identification of either of these regions independently or as a protein that contains both regions. Molecules with only a RII binding domain may act as an EBP and be subject to antibody mediated blockade by a vaccine or blocking therapy formulation.

Also included in the present invention are nucleotide sequences that encode each of the paralogs of *P. falciparum* erythrocyte binding protein described herein. The compositions further include vectors containing a DNA sequence encoding at least one of the paralogues of *P. falciparum*, fragments thereof, or blocking peptides, wherein the vectors are capable of expressing *P. falciparum* paralogues, fragments thereof, or blocking peptides when present in a cell. Cells containing the vectors are also included as compositions, wherein the vectors contain a DNA sequence encoding at least one of the paralogues of *P. falciparum*, fragments thereof, or blocking peptides, and wherein the vectors are capable of expressing at least one of the paralogues of *P. falciparum*, fragments thereof, or blocking peptides, when present in the cell.

The family of paralogues of the *P. falciparum* erythrocyte binding protein (EBP) and the blocking peptides described herein are useful *in vitro* as research tools for studying *P. falciparum* in general and *P. falciparum* related diseases such as malaria. The family of paralogues of *P. falciparum* EBA-175 are also useful as diagnostic reagents in the immunoassays described herein.

Additionally, the paralogues of EBA-175 and blocking peptides of the present invention are useful for the production of vaccines and therapeutic compositions. Pharmaceutical compositions containing a member of the paralogue family and/or peptides such as vaccines and therapeutic formulations are provided. The methods described herein are methods for detection, diagnosis, preventionand treatment of *P. falciparum* mediated malarial infections. Assays for the detection or quantitation of *P. falciparum* antigens may employ antigens derived from a biological sample such as a biological fluid or tissue or from culture media.

Additionally, antibodies specific for each of the paralogues are provided for in this invention. The compositions and uses disclosed above for the paralogues may include the antibodies and fragments thereof in place of, or in addition to, the paralogues.

The terms "a", "an" and "the" as used herein are defined to mean "one or more" and include the plural unless the context is inappropriate.

The terms “polypeptide”, “peptide”, and “protein”, as used herein, are interchangeable and are defined to mean a biomolecule composed of two or more amino acids linked by a peptide bond.

The term "antigen" refers to an entity or fragment thereof which can induce an immune response in a mammal. The term includes immunogens and regions responsible for antigenicity or antigenic determinants. "Antigenic determinant" refers to a region of a *P. falciparum* protein recognized by an antibody.

As used herein, the terms "detecting" or "detection" refer to quantitatively or quantitatively determining the presence of the biomolecule under investigation.

By "isolated" is meant a biological molecule free from at least some of the components with which it naturally occurs.

The terms "antibody" and "antibodies" as used herein include monoclonal antibodies, polyclonal, chimeric, single chain, bispecific, simianized, and humanized antibodies, Fab fragments, including the products of an Fab immunoglobulin expression library, and peptide antibody fragments.

The phrases "specific for", "specifically binds to", "specifically hybridizes to" and "specifically immunoreactive with", when referring to an antibody or blocking peptide, refer to a binding reaction which is determinative of the presence of a peptide or antibody in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies and blocking peptides bind preferentially to a particular peptide or antibody and do not bind in a significant amount to other proteins present in the sample. Specific binding to a peptide or antibody under such conditions requires an antibody or blocking peptide that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies and peptides specifically

immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. *See*, Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

As used herein, the term "paralogue" includes different genes in the same species which are so similar in nucleotide sequence or amino acid sequence or functional regions contained within conserved regions of each molecule that they are assumed to have originated from a single ancestral gene.

As used herein, the term "vaccine" includes compositions comprising the paralogues of the invention, blocking or neutralizing antibodies, or fragments thereof, or blocking peptides or fragments of the paralogues, used for passive immunization of individuals prior to or following infection by *P. falciparum*. Vaccines are known in the art and are used to stimulate immune response in the body by creating antibodies or activated T lymphocytes capable of controlling the infecting organism. The result is protection against a disease with the duration of the protection depending on the particular vaccine. The immune system produces antibodies and memory cells for pathogens so that subsequent exposure does not result in disease. A successful vaccine does the same thing, usually without risk of illness.

Vaccines can comprise four general classes: those containing infectious agents killed by physical or chemical means; those containing living attenuated infectious organisms; those containing soluble toxins or microorganisms; and those containing substances extracted from the infectious agents.

Means of administering vaccines include, but are not limited to, orally or parenterally by injection, preferably by subcutaneous or intramuscular injection. Preparation and administration of oral vaccines are disclosed in U.S. Patent No. 6,103,243, incorporated herein by reference.

The terms "blocking antibodies" or "neutralizing antibodies" refer to antibodies that bind specifically to *P. falciparum* antigens. The term "blocking peptides" refers to peptides that specifically inhibit the binding of*P. falciparum* to an erythrocyte. More particularly, the term "blocking peptides" refers to peptides that specifically inhibit the binding of an EBA-175 erythrocyte binding protein to an erythrocyte.

The compositions of the present invention include at least one member of a family of paralogues to*P. falciparum* erythrocytic binding protein EBA-175. *P. falciparum* erythrocytic binding proteins are*P. falciparum* derived proteins that bind to residues or proteins present on erythrocytes and facilitate *P. falciparum* invasion of erythrocytes.

The compositions of the present invention also include portions of the paralogues for use as blocking peptides that specifically inhibit binding of*P. falciparum* erythrocytic binding proteins to erythrocytes. In another preferred embodiment, the blocking peptides have a length within the range of 5-15 amino acids. Preferably, the length is within the range of 9-11 amino acids. In an alternate embodiment, there is a cysteine residue cap on each end of the blocking peptide. When creating the blocking peptides of the present invention, it is to be noted that the peptides may optionally comprise a carboxy-terminal amino acid sequence of GGGS (SEQ ID NO:5) as is well known in phage display techniques.

A preferred embodiment of the invention is the EBP2 paralogue of *P. falciparum* EBA-175. The EBP2

paralogue has a partial open reading frame (ORF) of a gene that shared the conserved cysteine rich motif of the EBP family and especially of EBA-175 RII (F1 and F2) although the overall amino acid identity was less than 25% (shown in Figure 2).

The ORF of the EPB2 gene sequence was compared to the *P. falciparum* 3D7 strain EBA-175 region I to VI. The overall level of amino acid identity was 24.7% by the Lipman-Pearson Method of analysis. A comparison of EBA-175 region II, identified as the ligand-receptor binding domain for its receptor glycophorin A, demonstrated almost complete conservation of the cysteine residues (26 out of 27). Comparison of RII alone by the Lipman-Pearson Method of analysis showed 37.9% level of conservation when identical amino acids and conserved substitutions were included. EBA-175 RII contains two domains that have similar cysteine motifs (F1 and F2). EBP2 has a similar structural arrangement, identified here as F1 and F2. The unique deletion of a cysteine residue was found in F1 (Fig. 2). Other structural amino acids, proline (6 out of 12) and tryptophan (13 out of 13) were also conserved. Another conserved region identified earlier within EBA-175 and DABP (Adams *et al.*, 1992 PNAS), with an unknown function, is RVI. Comparison of the EBP2 RVI and EBA-175 RVI deduced amino sequence showed that the cysteine residues were completely conserved (8 out of 8). The level of amino acid conservation (identity and conserved substitutions) was 41.6% by the same analysis as described above.

The gene *ebp2* is apparently located on chromosome 13. The ORF of the *ebp2* gene encodes for two regions that are conserved between *P. falciparum, P. vivax* and *P. knowlesi* (Adams *et al.*, 1992). The first region identified as

the ligand-binding domain is RII and the second region that has as yet an unknown function is RVI (Fig. 2). A comparison between EBP2 and EBA-175 showed that EBP2 has little homology with the regions III to V defined for EBA-175. The ORF of the partial gene sequence encoding *epb2* does not include the membrane-spanning domain nor the cytoplasmic tail described for EBA-175. The ORF of the partial gene sequence of *ebp2* encoded for a molecule of approximately 133 kDa. The molecular mass of EBP2 identified by immunoprecipitation was 130 kDa (Example 5), which suggests that nearly the entire gene sequence has been identified. Subcellular localization studies by IFA demonstrated that EBP2 colocalized with EBA-175 at the apical end of the merozoite. EBA-175 is trafficked to the micronemes, which are organelles localized at the merozoites apical end and are involved in parasite invasion (Sim *et al.*, 1992).

An orthologue of EBA-175 present in *P. vivax*, that is identified as the Duffy-antigen binding protein is most similar to EBP2-F1. A comparison of EBP2 and the *P. vivax* DABP region II deduced amino acid sequence showed that DABP region II is more similar to EBP2-F1 (12 out of 13 cysteines are conserved) which is similar to that previously reported for EBA-175 RII-F1 (Adams *et al.*, 1992).

Parasite invasion of erythrocytes is known to occur by different invasion pathways *in vitro*. Analysis of *P. falciparum* strains adapted to long-term *in vitro* culture (Narum *et al.*, 2000, Dolan *et al.*, 1994) has shown that different parasites strains may invade erythrocytes in a sialic acid dependent and sialic acid independent manner. This is generally determined by enzymatically treating erythrocytes with neuraminidase, which cleaves sialic acid residues. It is

known that EBA-175 binds sialic acid residues in conjunction with the peptide backbone of glycophorin A (Sim*et al.*, 1994). EBP2 erythrocyte binding was also dependent on sialic acid residues for binding (Example 6). The binding affinity of EBP2 appears greater than EBA-175 since EBP2 was not removed by 150 mM NaCl wash (Example 5). The existence of a family of EBPs broadens the spectrum of phenotypic differences between erythrocytes that that may be utilized by*P. falciparum*.

In summary, EPB2 is a novel *P. falciparum* 130 kDa EBP belonging to a family of paralogues of EBA-175. EBP2 also has a ligand-binding domain, identified as RII. EBP2 is localized within the merozoite apex and native protein binds erythrocytes in a sialic acid dependent manner. EBP2 is a malaria vaccine candidate and target for a receptor blocking therapy.

Also provided herein are antibodies to each of the paralogues. In a preferred embodiment of the present invention, the antibodies are specific for each of the paralogues of the *P. falciparum* binding proteins. In a further preferred embodiment, the antibodies are monoclonal and directed toward paralogues of the erythrocyte binding protein EBA-175 as defined by Camus and Hadley (1985), Sim*et al.* (1990), and Orlandi *et al.* (1992).

In another preferred embodiment EBP2 RII specific antibodies that did not cross-react with native EBA-175 were generated using a DNA vaccine. These antibodies recognize a novel 130 kDa protein that bound human erythrocytes in a sialic acid dependent manner. EBP2 RII specific antibodies blocked native EBP2 binding in a concentration dependent manner, which indicated that EBP2 RII was the ligand binding domain.

The inventors have studied whether certain EBA-175 RII paralogues that block EBA-175 binding will inhibit merozoite invasion *in vitro*. The inventors have also studied whether certain antibodies specific to EBA-175 paralogues have a similar blocking effect on merozoite invasion. It was found that EBP2 RII specific antibodies blocked EBP2 binding to erythrocytes. EBA-175 RII antibody titers correlate with control of parasitemia in an EBA-175 RII Aotus monkey challenge study. Therefore, EBP2 antibodies for inhibition of parasite development *in vitro* are claimed herein.

The antibodies of the present invention can be polyclonal antibodies or monoclonal antibodies. Antibodies specific for the family of paralogues of *P. falciparum* erythrocyte binding proteins may be administered to a human or animal to passively immunize the human or animal against *P. falciparum* infection, thereby reducing *P. falciparum* related diseases such as malaria. Antibody derived blocking peptides specific for *P. falciparum* erythrocyte binding proteins may be administered to a human or animal to immunize the human or animal against *P. falciparum* infection, thereby reducing *P. falciparum* related diseases such as malaria. The antibodies are also useful as *in vitro* research tools for studying malaria and for isolating large quantities of *P. falciparum* erythrocyte binding proteins. The antibodies specific for the family of paralogues of *P. falciparum* erythrocyte binding proteins can be used in diagnostic kits to detect the presence and quantity of *P. falciparum* erythrocyte binding proteins, which is diagnostic or prognostic for the occurrence or recurrence of diseases such as malaria. Additionally, the antibody derived blocking peptides that inhibit binding of *P. falciparum* binding to erythrocytes can be used in diagnostic kits to detect the presence and

quantity of *P. falciparum* antibodies, which is diagnostic or prognostic for the occurrence of diseases such as malaria.

When labeled isotopically or with other molecules or proteins, the *P. falciparum* antibodies to the family of EBA-175 paralogues are useful in the identification and quantitation of *P. falciparum* utilizing techniques including, but not limited to, positron emission tomography, autoradiography, flow cytometry, radioreceptor binding assays, and immunohistochemistry.

The antibodies and antibody derived blocking peptides of each of the paralogues of the present invention can be isolated from serum or synthesized by chemical or biological methods. For example, the antibodies and antibody derived blocking peptides can be isolated from cell culture, produced by recombinant gene expression or polypeptide synthesis, or derived by *in vitro* enzymatic catalysis of larger, encompassing polypeptides to yield blocking or neutralizing antibodies or antibody derived blocking peptides. Recombinant techniques include gene amplification from DNA sources using amplification techniques such as the polymerase chain reaction (PCR), and gene amplification from RNA sources using amplification techniques such as reverse transcriptase/PCR. In a preferred embodiment, the antibody derived blocking peptides are produced and analyzed via phage display technology. Phage vectors that may be used in phage display technology include, but are not limited to, λ, M13, MS2, Mu, P4, λgtII, and ϕX174.

The antibodies and antibody derived blocking peptides of the present invention may be labeled directly with a detectable label for identification and quantitation of *P. falciparum* or antibody thereto. Labels for use in

immunoassays are generally known to those skilled in the art and include enzymes, radioisotopes, and fluorescent, luminescent and chromogenic substances including colored particles such as colloidal gold and latex beads. Suitable immunoassays include enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays.

Alternatively, the antibodies and antibody derived blocking peptides of the present invention may be labeled indirectly by reaction with labeled substances that have an affinity for immunoglobulin, such as protein A or G or second antibodies. When using secondary antibodies, a suitable immunoassay is an immunoblot or Western blot. Additionally, the antibodies or antibody derived blocking peptides may be conjugated with a second substance and detected with a labeled third substance having an affinity for the second substance conjugated to the antibody. For example, the antibodies or antibody derived blocking peptides maybe conjugated to biotin and the antibody-biotin conjugate detected using labeled avidin or streptavidin. Similarly, the antibodies or antibody derived blocking peptides may be conjugated to a hapten and the antibody-hapten conjugate detected using labeled anti-hapten antibody. These and other methods of labeling antibodies and assay conjugates are well known to those skilled in the art.

When labeled with a detectable biomolecule or chemical, the *P. falciparum* erythrocyte binding protein antibodies and antibody derived blocking peptides described above are useful for purposes such as *in vivo* and *in vitro* diagnostics and laboratory research using the methods and assays described below. Various types of labels and methods of conjugating the labels to the polypeptides and antibodies are well known to those skilled in the art. Several specific labels are set forth below.

For example, the antibodies and antibody derived blocking peptides are conjugated to a radiolabel such as, but not restricted to, ^{32}P, ^{3}H, ^{14}C, ^{35}S, ^{125}I, or ^{131}I. Detection of a label can be by methods such as scintillation counting, gamma ray spectrometry or autoradiography.

Bioluminescent labels, such as derivatives of firefly luciferin, are also useful. The bioluminescent substance is covalently bound to the polypeptide or antibody by conventional methods, and the labeled antibody is detected when an enzyme, such as luciferase, catalyzes a reaction with ATP causing the bioluminescent molecule to emit photons of light.

Fluorogens may also be used as labels. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycocyanin, phycocyanin, rhodamine, and Texas Red. The fluorogens are generally detected by a fluorescence detector.

The antibodies and antibody derived peptides can alternatively be labeled with a chromogen to provide an enzyme or affinity label. For example, the antibody can be biotinylated so that it can be utilized in a biotin-avidin reaction, which may also be coupled to a label such as an enzyme or fluorogen. Alternatively, the antibodies or antibody derived peptides can be labeled with peroxidase, alkaline phosphatase or other enzymes giving a chromogenic or fluorogenic reaction upon addition of substrate. Additives such as 5-amino-2,3-dihydro-1,4-phthalazinedione (also known as Luminol™) (Sigma Chemical Company, St. Louis, MO) and rate enhancers such as p-hydroxybiphenyl (also known as p-phenylphenol) (Sigma Chemical Company, St. Louis, MO) can be used to amplify enzymes such as horseradish peroxidase through a luminescent reaction; and luminogeneic or

fluorogenic dioxetane derivatives of enzyme substrates can also be used. Such labels can be detected using enzyme-linked immunoassays (ELISA) or by detecting a color change with the aid of a spectrophotometer. In addition, antibodies and antibody derived peptides may be labeled with colloidal gold for use in immunoelectron microscopy in accordance with methods well known to those skilled in the art.

The paralogues, antibodies and derived blocking peptides described herein are particularly useful for the treatment, prevention, diagnosis and detection of*P. falciparum* infections. The paralogues, antibodies and derived blocking peptides of the present invention may be used for the treatment, prevention, diagnosis or prognosis of *P. falciparum* related diseases such as malaria. Methods of prevention include passive immunization with the paralogues and/or antibodies of the present invention prior to infection by *P. falciparum* to inhibit parasitic infection of erythrocytes. Methods of prevention also include active immunization with the derived blocking peptides of the present invention prior to infection by *P. falciparum* to inhibit parasitic infection of erythrocytes. Methods of treatment include administration of the paralogues, antibodies and/or derived blocking peptides after infection to inhibit the spread of the parasite and ameliorate the symptoms of *P. falciparum* infection. The paralogues, antibodies and derived peptides of the present invention may also be used to detect or quantify *P. falciparum* in a biological sample or specimen or culture media, or used in diagnostic methods and kits, as described below. Results from these tests can be used to predict or diagnose the occurrence or recurrence of *P. falciparum* mediated diseases such as malaria. Paralogues, antibodies and derived peptides of the invention may also be used in production facilities or laboratories to isolate additional

quantities of the *P. falciparum* erythrocytic binding proteins and/or paralogues thereof, such as by affinity chromatography, or for the development of peptide agonists or antagonists.

Plasmodium falciparum related diseases such as malaria are prevented or treated by administering to a patient suffering from a *P. falciparum* related disease, a pharmaceutical composition containing substantially purified *P. falciparum* erythrocyte binding protein paralogues, peptides thereof and antibodies thereof, *P. falciparum* antibody derived blocking peptides, *P. falciparum* polypeptide agonists or antagonists, or *P. falciparum* polypeptide antisera. Additional prevention and treatment methods include administration of*P. falciparum* erythrocyte binding protein paralogues, peptides thereof and antibodies thereof, *P. falciparum* antibody derived blocking peptides, *P. falciparum* polypeptide antisera, or *P. falciparum* polypeptide receptor agonists and antagonists linked to cytotoxic or anti-parasitic agents.

The paralogues and antibodies specific thereof may be administered to a patient to passively immunize the patient against *P. falciparum* infection, thereby reducing *P. falciparum* related diseases such as malaria. The derived blocking peptides that specifically inhibit binding of *P. falciparum* to a red blood cell may also be administered to a patient to actively immunize the patient against*P. falciparum* infection, thereby reducing *P. falciparum* related diseases such as malaria. Administration of the *P. falciparum* erythrocyte binding protein paralogues, antibodies or derived blocking peptides may occur prior to any signs of *P. falciparum* infection. Such an administration would be important in individuals in areas where *P. falciparum* is endemic, or to individuals planning to travel to endemic areas. Administration of the *P. falciparum* erythrocyte binding protein paralogues,

antibodies and derived blocking peptides may also occur after signs of *P. falciparum* infection have surfaced in order to interrupt the life cycle of the *Plasmodium* parasite and inhibit the spread of the organism.

In a preferred embodiment, a vaccine for passive or active immunization against malaria is packaged in a single dosage for immunization by parenteral (i.e., intramuscular, intradermal or subcutaneous) administration. The vaccine is most preferably injected intramuscularly into the deltoid muscle. The vaccine is preferably combined with a pharmaceutically acceptable carrier to facilitate administration. The carrier is usually water or a buffered saline, with or without a preservative. The vaccine may be lyophilized for re-suspension at the time of administration or in solution.

The carrier to which the paralogues, antibody or derived blocking peptides may be conjugated may also be a polymeric delayed release system. Synthetic polymers are particularly useful in the formulation of a vaccine to effect the controlled release of antibody. Microencapsulation of the paralogues, antibody or derived blocking peptide will also give a controlled release. A number of factors contribute to the selection of a particular polymer for microencapsulation. The reproducibility of polymer synthesis and the microencapsulation process, the cost of the microencapsulation materials and process, the toxicological profile, the requirements for variable release kinetics and the physicochemical compatibility of the polymer and the antigens are all factors that must be considered. Examples of useful polymers are polycarbonates, polyesters, polyurethanes, polyorthoesters polyamides, poly (d,l-lactide-co-glycolide) (PLGA) and other biodegradable polymers.

The preferred dose for human administration of the pharmaceutical composition or vaccine is from 0.01 mg/kg to 10 mg/kg. Based on this range, equivalent dosages for heavier body weights can be determined. The dose should be adjusted to suit the individual to whom the composition is administered and will vary with age, weight and metabolism of the individual. The vaccine may additionally contain stabilizers such as thimerosal (ethyl(2-mercaptobenzoate-S)mercury sodium salt) (Sigma Chemical Company, St. Louis, MO) or physiologically acceptable preservatives.

Region II DNA vaccines were constructed using the DNA vaccine backbone VR1020 for the panel of EBPs using standard molecular biological methods. EBP Region II gene inserts were amplified by PCR and verified by restriction enzyme mapping and DNA sequencing of the complete insert (*ebp2* and *ebp3*) or plasmid junctions (*ebp4* and *ebp5*).

Polyclonal anti-sera from groups of Balb/c immunized by DNA vaccination were tested for recognition of *Plasmodium falciparum* blood stage proteins by indirect immunofluorescence antibody test (IFAT) using methanol fixed parasitized erythrocytes.

VM92 cells were transiently transfected with the panel of EBP Region II DNA vaccines as described for EBP2 and culture supernatants were tested for secreted EBP Region II protein by immunoblot using homologous DNA vaccinated mouse antiserum.

TABLE 1 is a summary of EBP2, EBP3, EBP4, and EBP5 region II DNA vaccine construction, expression *in vitro* and generation of polyclonal anti-EBP2, EBP3, EBP4, and EBP5 antisera *in vivo*.

TABLE 1

Protein identifier	Gene identifier	Chromosome location	EBP Region RII DNA vaccine produced	Mice immunized	IFAT staining results	Immunoblot results
EBA-175 or SABP[1]	*eba-175 (or ebp1)*	7	Yes	Yes	Positive	Positive
EBP2	*ebp2*	13	Yes	Yes	Positive	Positive
EBP3	*ebp3*	13	Yes	Yes	Negative	Negative
EBP4	*ebp4*	4	Yes	Yes	Negative	Negative
EBP5	*ebp5*	1	Yes	Yes	Negative	Negative

The paralogues and antibodies of the present invention may also be used for the detection of *P. falciparum* peptides in biological samples or culture media. There are many techniques known in the art for detecting a component such as a polypeptide in a mixture and/or measuring its amount. Immunoassays, which employ antibodies that bind specifically to the polypeptide of interest, are one of the better known measurement techniques. Classical methods involve reacting a sample containing the polypeptide with a known excess amount of antibody specific for the polypeptide, separating bound from free antibody, and determining the amount of one or the other. Often the antibody is labeled with a reporter group to aid in the determination of the amount of bound analyte as described above. The reporter group or "label" is commonly a fluorescent or radioactive group or an enzyme.

An immunoassay is performed for the detection of *P. falciparum* in a sample as follows:

A sample is collected or obtained using methods well known to those skilled in the art. The sample containing the *P. falciparum* polypeptides to be detected may be obtained from an culture media or any biological source. Examples of

biological sources include blood serum, blood plasma, urine, spinal fluid, fermentation fluid, lymph fluid, tissue culture fluid and ascites fluid. The sample may be diluted, purified, concentrated, filtered, dissolved, suspended or otherwise manipulated prior to immunoassay to optimize the immunoassay results.

To detect *P. falciparum* polypeptides, the sample is incubated with one or more of the *P. falciparum* erythrocyte binding protein paralogue antibodies of the present invention. The antibody may be labeled or conjugated to a solid phase bead or particle as also described herein. The labeled antibody is then detected using methods well known to those skilled in the art. The term "detecting" or "detected" as used herein means using known techniques for detection of biologic molecules such as immunochemical or histological methods. Such methods include immunological techniques employing monoclonal or polyclonal antibodies to the peptides, such as enzyme linked immunosorbant assays, radioimmunoassay, chemiluminescent assays, or other types of assays involving antibodies known to those skilled in the art.

Current binding assay technology benefits from the diversity of detection systems developed that use enzyme-catalyzed chromogenic reactions, radionuclides, chemiluminescence, bioluminescence, fluorescence, fluorescence polarization and a variety of potentiometric and optical biosensor techniques.

Binding assays rely on the binding of analyte by analyte receptors to determine the concentrations of analyte in a sample. Analyte-receptor assays can be described as either competitive or non-competitive. Non-competitive assays generally utilize analyte receptors in substantial excess over the concentration of analyte to be determined in the assay.

Sandwich assays are examples of non-competitive assays, that comprise one analyte receptor frequently bound to a solid phase and a second analyte receptor labeled to permit detection. The analyte first binds to the analyte receptor bound to a solid phase and the second labeled analyte receptor is then added to facilitate quantitation of the analyte. Bound analyte can easily be separated from unbound reagents, such as unbound labeled first analyte receptors, due to the use of an analyte receptor bound to a solid phase.

Competitive assays generally involve a sample suspected of containing analyte, an analyte-analogue conjugate, and the competition of these species for a limited number of binding sites provided by the analyte receptor. Competitive assays can be further described as being either homogeneous or heterogeneous. In homogeneous assays all of the reactants participating in the competition are mixed together and the quantity of analyte is determined by its effect on the extent of binding between analyte receptor and analyte-conjugate or analyte analogue-conjugate. The signal observed is modulated by the extent of this binding and can be related to the amount of analyte in the sample. U.S. Patent No. 3,817,837 describes such a homogeneous, competitive assay in which the analyte analogue conjugate is a analyte analogue-enzyme conjugate and the analyte receptor, in this case a paralogue of EBA-175 or an antibody thereof, is capable of binding to either the analyte or the analyte analogue. The binding of the paralogue or antibody to the analyte analogue-enzyme conjugate decreases the activity of the enzyme relative to the activity observed when the enzyme is in the unbound state. Due to competition between unbound analyte and analyte analogue-enzyme conjugate for analyte-receptor binding sites, as the analyte concentration increases the amount of unbound analyte

analogue-enzyme conjugate increases and thereby increases the observed signal. The product of the enzyme reaction may then be measured kinetically using a spectrophotometer.

Heterogeneous, competitive assays require a separation of analyte analogue conjugate bound to analyte receptor from the free analyte analogue conjugate and measurements of either the bound or the free fractions. Separation of the bound from the free may be accomplished by removal of the analyte receptor and anything bound to it from the free analyte analogue conjugate by immobilization of the analyte receptor on a solid phase or precipitation. The amount of the analyte analogue conjugate in the bound or the free fraction can then be determined and related to the concentration of the analyte in the sample. Normally the bound fraction is in a convenient form, for example, on a solid phase, so that it can be washed, if necessary, to remove remaining unbound analyte analogue conjugate and the measurement of the bound analyte analogue conjugate or related products is facilitated. The free fraction is normally in a liquid form that is generally inconvenient for measurements. If multiple analytes are being determined in a single assay, the determination of the free fraction of analyte analogue conjugate for each analyte is made impossible if all are mixed in a single liquid unless the responses of the individual analyte analogue conjugates can be distinguished in some manner. However, detecting the free fraction of analyte analogue conjugate in assays that are visually interpreted is a distinct advantage because the density of the color developed in such assays is generally proportional to the analyte concentration over much of the range of analyte concentration.

In a preferred embodiment, the method for detecting and characterizing *P. falciparum* polypeptides

comprises taking a sample from a protein production lot. A determination of the presence of the immunodominant polypeptides can then be made using assay techniques that are well known to those skilled in the art and include methods such as Western blot analysis, radioimmunoasssay and ELISA assays.

In a second preferred embodiment, the method for detecting *P. falciparum* polypeptides comprises taking biological samples, such as fluids and tissues, from a mammal for the diagnosis or prognosis of malaria. The sample is preferably obtained from the blood, cerebrospinal fluid, urine or tissues of a mammal, preferably a human or simian. A determination of the presence of the immunodominant polypeptides can then be made using assay techniques that are well known to those skilled in the art and include methods such as Western blot analysis, radioimmunoasssay and ELISA assays.

A kit for detecting the presence and quantity of*P. falciparum* paralogues, antibodies thereof and/or derived peptides is also provided. The kit can be in any configuration well known to those of ordinary skill in the art and is useful performing one or more of the methods described herein for the detection of *P. falciparum* in biological samples or for the detection or monitoring of*P. falciparum* infection in a patient or carrier. The kits are convenient in that they supply many if not all of the essential reagents for conducting an assay for the detection of*P. falciparum* in a biological sample. The reagents may be premeasured and contained in a stable form in vessels or on a solid phase in or on which the assay may be performed, thereby minimizing the number of manipulations carried out by the individual conducting the assay. In addition, the assay may be performed simultaneously with a standard that is included

with the kit, such as a predetermined amount of a paralogue of the invention, or antigen or antibody thereof, so that the results of the test can be validated or measured.

In one embodiment, the kit preferably contains one or more *Plasmodium falciparum* erythrocyte binding protein antibodies that can be used for the detection of *P. falciparum* binding proteins in a sample. The kit can additionally contain the appropriate reagents for binding or hybridizing the antibodies to their respective *P. falciparum* binding molecules or ligands in the sample as described herein and reagents that aid in detecting the bound peptides. The kit may additionally contain equipment for safely obtaining the sample, a vessel for containing the reagents, a timing means, a buffer for diluting the sample, and a colorimeter, reflectometer, or standard against which a color change may be measured.

In another preferred embodiment, the reagents, including the antibody, are lyophilized, most preferably in a single vessel. Addition of aqueous sample to the vessel results in solubilization of the lyophilized reagents, causing them to react. Most preferably, the reagents are sequentially lyophilized in a single container, in accordance with methods well known to those skilled in the art that minimize reaction by the reagents prior to addition of the sample.

The assay kit includes but is not limited to reagents to be employed in the following techniques; competitive and non-competitive assays, radioimmunoassay, bioluminescence and chemiluminescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including immunoblots and ELISAs, and immunocytochemistry. Materials used in conjunction with these techniques include, but are not limited to, microtiter plates, antibody coated strips or dipsticks for rapid monitoring

of urine or blood. For each kit, the range, sensitivity, precision, reliability, specificity and reproducibility of the assay are established. Intraassay and interassay variation is established at 20%, 50% and 80% points on the standard curves of displacement or activity.

In a further preferred embodiment, the assay kit uses immunoblot techniques and provides instructions, *P. falciparum* polypeptides, and *P. falciparum* erythrocyte binding protein antibodies conjugated to a detectable molecule. The kit is useful for the measurement of *P. falciparum* in biological fluids and tissue extracts of animals and humans with and without malaria, as well as in culture media.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims. All patents and references disclosed herein are incorporated by reference.

Example 1

Materials and methods for detecting P. falciparum EBPs

Genomic Database

The sequence data for *P. falciparum* chromosome 13 was obtained from The Sanger Centre website at http://www.sanger.ac.uk/Projects/P_falciparum/. Sequencing of *P. falciparum* chromosome 13 was accomplished as part of

the Malaria Genome Project with support by The Wellcome Trust.

Parasites

Plasmodium falciparum 3D7 strain (clone of NF54, Amsterdam Airport, human challenge strain) and FVO strain (Aotus adapted) were maintained as previously reported (Vernes et al., 1984). When appropriate, schizonts were purified on Percoll density gradient. The 3D7 parasites were metabolically labeled with TRAN^{35}S-LABEL™ (ICN Radiochemicals, Irvine, CA) as previously described (Sim *et al.*, 1994b). Essentially 2 x 10^8 parasites in 10 ml RPMI-1640 culture media deficient in methionine and cysteine were incubated with 1 mCi TRAN^{35}S-LABEL™ for 4 hours for parasite-cell pellets and 16-24 hours for culture supernatants. Parasitized erythrocytes for preparation of schizont extracts were washed twice in RPMI-1640 and cell pellets were frozen at –70°C. For the collection of labeled parasite proteins, cells were removed by centrifugation (1 min. at 20,000 x *g*) and supernatants were stored at -70°C.

RT-PCR analysis

Aliquots of purified mRNA, isolated from purified schizont infected erythrocytes using a mRNA isolation kit (Stratagene, La Jolla, CA), were stored precipitated in ethanol with 3M sodium acetate at –70°C. The mRNA was treated with DNAase to ensure that it was free of genomic DNA; the absence of DNA was confirmed by the lack of amplification in RT-PCR studies in the absence of reverse transcriptase. First strand cDNA transcripts were prepared using a poly dT primer from a cDNA CYCLE™ kit (Invitrogen, Carlsbad, CA). This first strand product was amplified by PCR using the

oligonucleotide forward primer 5' CAAGGAGAATGTATGGAAAGTA 3' and reverse primer 5' ATCTTCATATTCATTTGGACTCT 3'. The PCR amplified product was detected by ethidium bromide staining a 1% agarose gel.

EBP2 DNA sequence analysis and plasmid vaccine construction

P. falciparum EBP2 RII (amino acids 147-762, 1848bp) was amplified using AdvanTaq Plus™ DNA polymerase (Clontech, Palo Alto, CA) from 100 ng of 3D7 genomic DNA using the forward primer 5'ATGCGGATCCCAATATACGTTTATACAGAAACGTAC TC 3' and reverse primer 5'ATGCGGATCCTCATATATCGTGTTTTGTTTTAGG 3' which both contained a BamHI site and the reverse primer contained an additional internal stop codon for cloning into the shuttle vector PCR-Script™ as described by the manufacturer's instructions (Stratagene, La Jolla, CA). The *ebp2* RII gene fragment excised with BamHI and cloned into the expression plasmid vector VR1020 (identified as pEBP2-RII). The VR1020 plasmid vector utilizes the human cytomegalovirus promoter and intron A, and human tissue plasminogen activator as the secretory signal and the bovine growth hormone transcriptional terminator/polyadenylation signal (Hartikka *et al.*, 1996). A clone was selected for correct orientation by restriction-enzyme mapping. Both the forward and reverse strands of the *ebp2* RII ORF were sequenced using primers off of the vector and primers based on the cloned sequences (Veritas, Inc., Rockville, MD). Human melanoma cells (UM449), were transiently transfected with the plasmids

pEBP2RII, 3D7 encoded pEBA-175RII and VR1020 plasmid with Lipofectamine™ following the manufacture's protocol (Life Technologies, Gaithersburg, MD). Secretion of RII protein in culture supernatants was confirmed by Western blot. Plasmids were prepared for immunizations using an EndoFree Plasmid Giga kit (Qiagen, Valencia, CA). Purity was gauged by UV spectroscopy (260nm/280nm was between 1.70 and 1.90), agarose gel electrophoresis showing predominately supercoiled plasmid and endotoxin levels (<10 EU/mg) were detected using the *Limulus amebocyte* assay. The *ebp2* RVI was amplified by PCR as above except used Vent DNA polymerase (New England BioLabs, Beverly, MA) using the forward primer 5' TCTAGAGATACTAAAAGAGTAAGG 3' and reverse primer 5' TGATTGACCCTCGCTTTTAAAAC 3'. The PCR amplified fragment was gel purified and both the forward and reverse strands were sequenced directly (Veritas, Inc.).

Animals and immunizations

All animal studies were done in compliance with protocols approved by Animal Care and Use Committees. BALB/c mice were inoculated intradermally with a 29 gauge needle at two sites in the tail with a total of 50 μg VR1020 (empty vector), pEBP2RII in 50 μl PBS. The mice were inoculated on days 0, 21, 42 and bled approximately two weeks after each immunization. A fourth dose was administered approximately three months after the third dose and bled two weeks later. Pooled sera were assessed for antibodies to parasitized erythrocytes by IFA.

Immunoprecipitation, immunoblotting and immunofluorescence assay

Aliquots of approximately 2 x 10^8 parasitized RBCs that had been metabolically labeled were extracted in buffer containing 1% Triton X-100 (Sigma, St. Louis, MO) (Narum *et al.*, 1994). Schizont infected erythrocyte lysates (50 to 75 µl) were immunoprecipitated with mouse serum or purified rabbit IgG coupled to Protein G (Pharmacia Biotech., Uppsala, Sweden) and the precipitates were washed as previously described using a buffer containing Triton X-100 (Deans and Jeans, 1987; Narum *et al.*, 1994). The labeled proteins were resolved by SDS-PAGE and detected and/or quantified with a phosphoimager (BioRad Molecular Imager FX, Hercules, CA). Immunoblots were prepared essentially as described previously (Narum et al) using EBP specific antisera. IFA on thin films containing schizont-infected erythrocytes used mouse anti-EBP2 sera (1/50 dilution in PBS-1%FCS) and rabbit anti-EBA-175 RII IgG (2 µg/ml in buffer) were co-incubated for 1 hour in a moisture chamber. After washing in PBS, the parasitized cells were co-incubated with species specific fluorescein or Alexa™ 546 labeled secondary antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD and Molecular Probes, Eugene, OR, respectively). The same microscope field was photographed using excitation for both fluorescein and Alexa 546. Alexa 546 emission appears orange.

EBPs binding studies and blocking of binding to erythrocytes

Metabolically labeled parasite culture supernatant containing [^{35}S]-metabolically labeled EBPs was used for erythrocyte binding assays as previously described by Camus

and Hadley (1985) or Sim *et al.* (1994). Briefly, two samples were prepared containing 1 ml [^{35}S]-labeled culture supernatant and 2 x 10^9 packed erythrocytes that were incubated for 45 minutes at room temperature while rocking for binding to occur. To one vial, the erythrocytes were pelleted, washed thrice in PBS and the erythrocyte pellet was extracted in 500 µl 1% Triton X-100 extraction buffer. EBP bound erythrocyte lysates were immunoprecipitated using 80 µl extract with antigen specific antisera or control sera coupled to Protein G. The other sample was divided into four parts and the EBPs were eluted from erythrocytes with 9 µl of RPMI 1640, 1.5 M NaCl, 10% FCS, and 2 mM phenymethylsulfonyl fluoride as previously described (Sim *et al.*, 1994). Elution material was pooled and diluted 15-fold in PBS-1%FCS and equal volumes were immunoprecipitated with antigen specific antisera or control sera as above. Gels were dried and quantified with a phosphoimager (BioRad Molecular Imager FX, Hercules, CA).

Blocking of binding was performed by pre-incubating a titration of EBP2 specific antisera with 100 µl [^{35}S]-labeled culture supernatant for 45 minutes and then 2 x 10^8 erythrocytes were added and incubated for 30 minutes at room temperature while rocking. Erythrocyte pellets were washed thrice with PBS, extracted in 50 µl 1% Triton X-100 extraction buffer, immunoprecipitated with EBP2 antisera coupled to Protein G and analyzed as above. Blocking of binding was calculated as follows: (control – experiment/control) x 100. The "control" is the counts from EBP2 bound to erythrocytes in the presence of control sera.

Neuraminidase treatment of erythrocytes:

Human blood was collected in a final 10% citrate phosphate dextrose solution, washed and treated with 0.2 U per 1 x 10^9 erythrocytes Vibrio cholerae neuraminidase (Gibco BRL, Gaithersburg, MD) as previously described (Liang et al 2000).

Example 2

Expression of EBP2 in parasitized erythrocytes

To examine whether *ebp2* was transcribed a mRNA transcript was detected with RT-PCR. Parasite mRNA isolated from 3D7 schizont-infected erythrocytes was used for first strand synthesis with random primers or with a poly dT primer and then PCR amplified with an *ebp2* primer pair or *eba-175* primer pair as a control. Appropriate size DNA fragments were detected on an ethidium bromide stained agarose gel for *ebp2* and *eba-175*. Using genomic 3D7 DNA, *ebp2* RII was amplified by PCR and cloned into a naked DNA vaccine plasmid (Hartikka *et al.*, 1996). Both the forward and reverse strands of the DNA fragment were sequenced. A single point nucleotide change at position 1654 (A to G) encoded an amino acid substitution from Asn to Asp. This single amino acid difference is the result of a PCR-introduced artifact.

Recombinant EBP2 RII and EBA-175 RII derived from supernatants by transient transfection of UM449 cells were immunoblotted with specific antisera generated as below.

A western blot of secreted *P. falciparum* 3D7 EBP2 RII (A) and 3D7 EBA-175 RII (B) proteins showed expression of EBP2 *in vitro* and specificity of EBP2 and EBA-175 antibodies. UM449 cells were transiently transfected with naked DNA plasmid pEBP2 RII and run in lane 1. VR1020

(Vical, San Diego, CA) control was run in lane 2. pEBA-175 RII was run in lane 3. EBP2 RII and EBA-175 RII recombinant proteins were detected by RII specific mouse antibodies. EBP2 RII and EBA-175 RII anti-sera were specific for self and showed no detectable cross-reactivity.

Pooled immune sera from BALB/c mice immunized with the EBP2 RII DNA vaccine and empty vector as control were tested for the presence of EBP2 RII specific antibodies by Immunofluorescence assay (IFA). The IFA showed co-localization of EBP2 and EBA-175 within *P. falciparum* schizont infected erythrocyte. EBP2 was stained with mouse anti-EBP2 RII sera and EBA-175 was stained with rabbit anti-EBA-175 RII IgG. Both primary and secondary antibody controls were negative for staining. Cells were magnified 1000-fold. EBP2 RII antisera recognized 3D7 and FVO schizont-infected erythrocytes and gave a punctate apical pattern of fluorescence. The reciprocal end-point titer of pooled immune sera was 1600 on 3D7 parasitized erythrocytes by IFA. The subcellular pattern of apical fluorescence was compared to EBA-175 using EBA-175 RII specific antibodies generated in rabbits. The results showed that EBP2 and EBA-175 colocalized to the same subcellular location within the merozoite's apex.

Example 3

EBP2 RII specific antibodies recognize a 130 kDa P. falciparum protein

To determine the molecular mass of EBP2 [^{35}S]-metabolically labeled *P. falciparum* 3D7 strain schizont-infected erythrocyte lysates were incubated with EBP2 RII

specific antibodies coupled to Protein G-sepharose. [^{35}S]-labeled *P. falciparum* schizont-infected erythrocyte lysate were immunoprecipitated and human erythrocyte bound [^{35}S]–labeled EBP2 were detected from [^{35}S]–labeled parasite culture supernatants. EBA-175 specific polyclonal rabbit IgG was included as a control. Results were obtained with EBP2 specific polyclonal sera or control and EBA-175 specific polyclonal IgG and control. [^{35}S]-labeled EBP2 and EBA-175 were immunoprecipitated from lysates of erythrocytes with bound EBPs. [^{35}S]-labeled EBP2 and EBA-175 were eluted off human erythrocytes with 1M NaCl and then immunoprecipitated with EBP2 or EBA-175 specific antibodies. Mouse and rabbit adjuvant controls and molecular mass markers were employed. The molecular mass of processed or degraded forms of EBP2 were: a, 117.2; b, 92.2; c, 85.8; and d, 697 kDa. EBP2 RII antibodies immunoprecipitated a 130 kDa molecule as determined by SDS-PAGE under reducing conditions. The theoretical molecular mass of the *ebp2* ORF is 133,018 Daltons hence the observed molecular mass is similar to the theoretical. EBA-175 was also immunoprecipitated with EBA-175 RII specific rabbit polyclonal IgG as a control. The EBP2 RII antisera did not immunoprecipitate the abundantly labeled EBA-175 nor did EBA-175 RII antibodies immunoprecipitate EBP2.

Example 4

EBP2 binds human erythrocytes

It was determined that EBP2 bound to human erythrocytes by using [^{35}S]-labeled *P. falciparum* culture supernatants that contained *P. falciparum* proteins released during maturation of schizogony *in vitro*. Immunoprecipitation

of human erythrocyte lysates or eluates prepared as described in Materials and Methods showed that EBP2 bound human erythrocytes. EBA-175 specific rabbit IgG and rabbit control IgG were included in the analysis. The binding affinity of EBP2 was greater than that of EBA-175. Immunoprecipitation of erythrocyte lysates that were prepared by incubating erythrocytes and [^{35}S]-labeled *P. falciparum* culture supernatants, washed with PBS and incubated with mouse EBP2 RII and rabbit EBA-175 RII specific antibodies showed that EBP2 was not removed by the PBS while EBA-175 was removed. EBP2 RII antisera predominately immunoprecipitated a 69.7 kDa fragment although other larger fragments were also detected but were only weakly visible. The 69.7 kDa fragment identified in the culture supernatant appears to be a processed or degraded product of the larger 130 kDa form of EBP2 identified in schizont-infected erythrocyte lysates. Analysis of erythrocyte eluates obtained by immunoprecipitation showed similar binding patterns for EBP2, although the intensity of the other fragments by this approach was greater. The EBP2 fragments were 117.2, 92.2, 85.8 and 69.7 kDa. EBA-175 was detected using these conditions as previously reported (Orlandi *et al.*, 1990).

Example 5

EBP2 RII specific antibodies block EBP2 erythrocyte binding

To determine whether EBP RII antibodies blocked EBP2 binding to erythrocytes, EBP2 immune sera was titrated with [^{35}S]-labeled *P. falciparum* culture supernatants, which was then incubated with human erythrocytes, pelleted, washed and extracted in extraction buffer. Control antisera was at a single dilution of 1/10. Equal volumes of erythrocyte lysate

were immunoprecipitated with EBP2 specific polyclonal antibodies. Immunoprecipitation of the erythrocyte lysates demonstrated that EBP2 RII specific antibodies blocked EBP2 binding to human erythrocytes (Figure 6). The ED_{50} blocking titer was between 1/160 and 1/640.

Example 6

EBP2 binding to human erythrocytes is dependent on sialic acid residues

Human erythrocytes were used in untreated form or were enzymatically treated with neuraminidase, which cleaves sialic acid residues. The erythrocytes were then incubated with [^{35}S]-labeled *P. falciparum* culture supernatants. Red blood cells (RBCs) were pelleted, washed and extracted in extraction buffer. Equal volumes of erythrocyte lysate were immunoprecipitated with EBP2 specific polyclonal antibodies. EBP2 bound the untreated erythrocytes. Human erythrocytes devoid of sialic acid residues (i.e., neuraminidase treated erythrocytes) did not bind the 69.7 kDa fragment of EBP2. The sialic acid binding results were similar when we tested for EBP2 binding using an EBA-175 erythrocyte binding assay.

Example 7

Preparation of vaccines using EBP3

DNA vaccine that encoded region II of EBP3 was constructed similarly to EBP2 region II. Forward primer for EBP3: 5' ATGC GGA TCC GAA AAG AAT AAA TTT ATT GAC ACT 3' BamHI; Reverse primer for EBP3: 5' ATGC GGA TCC TCA AGG AAA CAC ATT CGT TTT TAT AGG 3' BamHI. Mice were immunized and polyclonal immune sera

were tested for recognition of parasite proteins by IFAT on methanol fixed parasitized erythrocytes. The results are shown in the Table 1 above. The IFAT results were all negative for detection of an expressed parasite protein except for the novel protein EBP2. EBA-175 was used as a positive control in these studies. EBP3 polyclonal immune was also tested by immunoblot against supernatant collected from VM92 cells transiently transfected individually with the EBP DNA vaccine. The results were all negative except for EBP2 for self-recognition of a transiently expressed region II fragment of EBP2. Although the results for EBP3 was negative here, these studies do not exclude the expression of this protein in erythrocytic stage parasites or in other parasite stages e.g., sporozoite, exo-erythrocytic, or sexual stage parasites.

Example 8

Preparation of vaccines using EPB4

DNA vaccine that encoded region II of EBP4 was constructed by directly cloning EBP4 into the DNA vaccine VR1020. Specific forward and reverse primers with BamHI restriction sites were used to PCR amplify from genomic (P. falciparum) DNA (Fig. 2) using standard molecular biological techniques that are known in the art. Forward primer for EBP4: 5' ATGC GGA TCC AAT CTG AAA GCT CCA AAT GCT AAA TCC 3' BamHI; Reverse primer for EBP4: 5' ATGC GGA TCC TCA TAT AGG AAA CAC ATT CGT TTT TAT AGG 3' BamHI. Mice were immunized and polyclonal immune sera were tested for recognition of parasite proteins by IFAT on methanol fixed parasitized erythrocytes. The results are shown in the Table 1 above. The IFAT results were all negative for detection of an expressed parasite protein except

for the novel protein EBP2. EBA-175 was used as a positive control in these studies. EBP4 polyclonal immune was also tested by immunoblot against supernatant collected from VM92 cells transiently transfected individually with the EBP DNA vaccine. The results were all negative except for EBP2 for self-recognition of a transiently expressed region II fragment of EBP4. Although the results for EBP4 was negative here, these studies do not exclude the expression of this protein in erythrocytic stage parasites or in other parasite stages such as sporozoite, exo-erythrocytic, or sexual stage parasites.

Example 9

Preparation of vaccines using EPB5

DNA vaccine that encoded region II of EBP5 was constructed by directly cloning EBP5 into the DNA vaccine VR1020. Specific forward and reverse primers with BamHI restriction sites were used to PCR amplify from genomic (P. falciparum) DNA (Fig. 2) using standard molecular biological techniques that are known in the art. Forward primer for EBP5: 5' ATGC GGA TCC AAT AGA AAT AGT TTT GTT CAA 3' BamHI; Reverse primer for EBP5: 5' ATGC GGA TCC TCA TGA GTC TAT AGA TAA CAT TTC 3' BamHI. Mice were immunized and polyclonal immune sera were tested for recognition of parasite proteins by IFAT on methanol fixed parasitized erythrocytes. The results are shown in the Table 1 above. The IFAT results were all negative for detection of an expressed parasite protein except for the novel protein EBP2. EBA-175 was used as a positive control in these studies. EBP5 polyclonal immune was also tested by immunoblot against supernatant collected from VM92 cells transiently transfected individually with the EBP DNA vaccine. The results were all

negative except for EBP2 for self-recognition of a transiently expressed region II fragment of EBP2. Although the results for EBP5 was negative here, these studies do not exclude the expression of this protein in erythrocytic stage parasites or in other parasite stages such as sporozoite, exo-erythrocytic, or sexual stage parasites.

Modifications and variations of the present method will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims. All patents, patent applications and publications cited in this application are herein incorporated by reference as if each were incorporated individually.

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